

SoxF factors induce Notch1 expression via direct transcriptional regulation during early arterial development

Running title: SoxF induces Notch1 expression

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Summary statement

We report a novel arterial-specific regulatory element that modulates *Notch1* gene expression. The transcription factor family SOXF directly controls this enhancer activity during both fish and mouse embryogenesis.

Abstract

Arterial specification and differentiation are influenced by a number of regulatory pathways, encompassing numerous growth factors, signaling molecules and transcription factors. While it is known that the Vegfa-Notch cascade plays a central role in this biological process, the transcriptional hierarchy controlling arterial specification has not been fully delineated. To elucidate the direct transcriptional regulators of Notch receptor expression in arterial endothelial cells, we used histone signatures, DNaseI hypersensitivity and ChIP-seq data to identify enhancers for the human *NOTCH1* and zebrafish *notch1b* genes. These enhancers were able to direct arterial endothelial cell-restricted expression in transgenic models. Genetic disruption of SOXF binding sites clearly established a requirement for members of the SOXF group of transcription factors (SOX7, -17 and -18) to drive these enhancers activity *in vivo*. Further, endogenous deletion of the *notch1b* enhancer led to a significant augmentation of arterio-venous defects in notch-pathway deficient zebrafish. Loss of SoxF function revealed that these factors are necessary for the activity of *NOTCH1* and *notch1b* enhancers, and for correct endogenous Notch1 gene transcription. These findings therefore position SOXF transcription factors directly upstream of Notch receptor expression during the acquisition of arterial identity in vertebrates.

Introduction

Genetic specification of arterial fate has long been attributed to regulation downstream of the Vegfa and Notch pathways. Vegfa signaling is essential for arterial specification in both zebrafish and mammalian models, at least partially by stimulating the expression of components of the Notch pathway, while activation of Notch signaling can rescue defects in Vegfa deficient zebrafish embryos (Lanahan et al., 2010; Lawson et al., 2002; Liu et al., 2003; Visconti et al., 2002). Notch receptors Notch1 and Notch4, and the delta-like ligands Dll1, Dll4, Jag1 and Jag2 are expressed in endothelial cells, where they play crucial roles in both arteriogenesis and angiogenesis (Lawson et al., 2002; Liu et al., 2003; Phng and Gerhardt, 2009; Roca and Adams, 2007). Ligand binding to the Notch receptor releases an intracellular domain (NICD) which translocates to the nucleus and forms a transcriptional activating complex with the otherwise repressive DNA-bound Rbpj (CSL, Su(H)) (Bray, 2006). Combined ablation of Notch1 and 4, both principally expressed in arterial endothelial cells during early vascular remodeling (Chong et al., 2011; Espen D Jahnsen et al., 2015) results in severe vascular remodeling defects (Krebs et al., 2000), as does the ablation of Notch downstream effector Rbpj and Delta-like ligand 4 (Dll4), a Notch ligand specific to arteries within the vasculature (Duarte et al., 2004; Gale et al., 2004; Krebs et al., 2004). However, loss of Notch signaling does not fully recapitulate the arterial defects downstream of Vegfa ablation (Carmeliet et al., 1996; Krebs et al., 2000; Lawson et al., 2002), and the arterially restricted gene expression pattern of components of the Notch pathway do not fully overlap with activated Vegfa (Lawson, 2003), suggesting that additional factors are involved in the regulation of Notch-mediated arterial fate.

The SoxF group of transcription factors (Sox7, -17 and -18), are expressed in endothelial cells from early in development (Francois et al., 2010). While each SoxF member displays a subtly different endothelial expression pattern, all three factors are expressed early in arterial development (Corada et al., 2013; François et al., 2008; Zhou et al., 2015) and share considerable functional redundancy, complicating the interpretation of the consequences of gene disruption (Hosking et al., 2009; Zhou et al., 2015). Combined loss of *sox7* and *sox18* in zebrafish, both by morpholino-based knockdown and genetic mutation, resulted in serious arterio-venous malformations similar to those seen after Notch ablation, suggesting that SoxF factors may genetically interact with the Notch pathway in endothelial cells (Cermenati et al., 2008; Hermkens et al., 2015; Herpers et al., 2008; Lawson et al., 2001). Further evidence was provided in mice, where endothelial-specific ablation of

Sox17 caused arterial differentiation and remodeling defects (Corada et al., 2013), and conditional deletion of SoxF factors in the adult resulted in loss of major vessel identity in the retina (Zhou et al., 2015). Inhibition of Vegf signaling can significantly impact SoxF expression and activity in both mouse and zebrafish models (Kim et al., 2016; Pendeville et al., 2008; Duong et al., 2014; Pennisi et al., 2000b), suggesting that members of the SoxF family lie downstream of Vegfa. Further, SoxF acts in a positive feed-forward loop to maintain *Flk1* expression (Kim et al., 2016). By contrast, the ablation of Notch signaling in the vasculature does not significantly impact the expression of SoxF (Abdelilah et al., 1996; Corada et al., 2013). Different experimental models have positioned SoxF genes both upstream (Corada et al., 2013) and downstream (Lee et al., 2014) of Notch signaling in endothelial cells, suggesting a complex relationship between these two pathways.

Analysis of the only known enhancers for the Notch pathway, the arterial-specific *Dll4*-12 (Sacilotto et al., 2013) and *Dll4*_{in3} enhancers (Sacilotto et al., 2013; Wythe et al., 2013), has demonstrated a critical role for SoxF factors in the regulation of the Notch ligand *Dll4* expression in arteries, in combination with Rbpj/Notch binding and in the presence of Ets factors including Erg (Sacilotto et al., 2013; Wythe et al., 2013). Although this work clearly positioned SoxF and Notch pathways as crucial regulators of arterial specification, and is supported by analysis placing *Sox17* upstream of Notch signaling in mouse models (Corada et al., 2013), ablation of arterial marker expression only occurs after the removal of both SoxF factors and Notch signaling in combination (Sacilotto et al., 2013). Consequently, the precise transcriptional hierarchy of SoxF and Notch has yet to be fully established. While studies of the Notch receptor genes *Notch1* and *4* also identified SoxF binding motifs within putative promoter sequences (Corada et al., 2013; Lizama et al., 2015), a requirement for these SoxF motifs in arterial-specific gene expression of Notch receptors has not been established. In this study, we have used the *in vivo* identification and characterization of arterial-specific enhancers directing *Notch1/notch1b* gene expression to demonstrate a direct requirement for SoxF factors in the transcriptional regulation of Notch receptors during early arterial differentiation, positioning SoxF factors upstream of Notch in the acquisition of arterial cell identity.

Results

Identification of an arterial-specific NOTCH1 intronic enhancer

Previous studies into the transcriptional regulation of the Notch receptors have not extended to the identification or analysis of gene enhancers (cis-regulatory elements) (Corada et al., 2013; Lizama et al., 2015; Wu et al., 2005). We therefore conducted a detailed *in silico* analysis of the *NOTCH1* locus with the aim of identifying novel, arterial-specific enhancers. This analysis focused on human *NOTCH1* in order to take advantage of the wealth of publically available information describing chromatin modifications in human endothelial cell lines. Using this information, we were able to pinpoint four regions of DNA rich in endothelial cell-specific H3K4Me1 and H3K27Ac histone modification and DNaseI digital genomic footprints, all marks closely associated with enhancer activity (Heintzman and Ren, 2009; Sabo et al., 2004) (Fig. 1A and Fig. S1). The putative enhancer regions were named *NOTCH1+33*, *NOTCH1+16*, *NOTCH1+3/5* and *NOTCH1-68* to reflect their distance from the transcriptional start site (TSS) in kb. Each enhancer region was cloned upstream of the silent *hsp68* minimal promoter and the *LacZ* reporter gene (Fig. 1B) and tested for its ability to drive reporter gene expression specifically to arterial endothelial cells of transient transgenic mice at embryonic stage 12-13 (E12-13). While each of the four putative enhancer regions were able to drive detectable levels of *LacZ* in transgenic mice, this expression was primarily neural, an expression pattern commonly seen when using the *hsp68* minimal promoter (for example (Becker et al., 2016; Sacilotto et al., 2013)) (Fig. 1C and S2). Only the *NOTCH1+33* and *NOTCH1+16* enhancers were able to direct expression to endothelial cells (Fig. 1C and S2). In the case of *NOTCH1+33*, vascular expression was detected in only one of the five transgenic mice. This expression was not restricted to the arterial endothelium but rather displayed a pan-endothelial cell expression (Fig. 1C and S2). This agrees with previous reports indicating that the mouse orthologue of this region, termed *Notch1_enh1*, also directed occasional vascular enhancer activity, but did not show arterial-specific expression (Zhou et al., 2017). Conversely, the 274-bp *NOTCH1+16* enhancer was able to robustly direct expression specifically to arterial endothelial cells within the vasculature at E12 in multiple independent transgenic embryos (Fig. 1C, Fig. 2 and Fig. S2). This indicates that the *NOTCH1+16* enhancer represent a novel, arterially-restricted enhancer within the *NOTCH1* locus. Analysis of a stable mouse line expressing the *NOTCH1+16:LacZ* transgene clearly demonstrated that the *NOTCH1+16* enhancer was strongly active from the very early stages of vascular development, mimicking the expression of endogenous Notch by

becoming arterial-restricted by late E9.5 and then maintaining an arterial endothelial cell-restricted expression pattern throughout embryonic development (Chong et al., 2011; Espen D Jahnsen et al., 2015) (Fig. 2).

The *NOTCH1+16* enhancer is bound and regulated by SoxF factors

To identify the transcription factors that potentially regulate the *NOTCH1+16* enhancer, we performed a ClustalW analysis of orthologous mammalian sequences (Fig. 3A). This analysis clearly identified nine conserved core consensus ETS binding motifs (GGAW,(Hollenhorst et al., 2011)), and two consensus SOX binding motifs (WWCAAW,(Mertin et al., 1999)) (Fig. 3A) within the 274bp *NOTCH1+16* enhancer. Because not all *in silico* consensus binding motifs are able to functionally bind the cognate protein, these motifs were then tested using electrophoretic mobility shift assay (EMSA). Both SOXF motifs (hmSOX-a and hmSOX-b) were able to bind recombinant SOX7 and SOX18 proteins in EMSA analysis (Fig. 3B), and three ETS motifs (hmETSa, b and c) were able to bind the endothelial Ets protein ETV2 (Fig. 3C). ETS motifs are common to all endothelial-expressed gene enhancers (De Val and Black, 2009). Although the ETS factor Erg has been implicated in arterial specification (Wythe et al., 2013), ETS motifs were unable to direct expression of the arterial-specific *Dll4* and *Flk1* enhancers without additional transcription factor binding motifs (Becker et al., 2016; Sacilotto et al., 2013; Wythe et al., 2013), suggesting that ETS factors alone are unlikely to regulate the *NOTCH1+16* enhancer. To test whether the SOXF motifs were playing a role in *NOTCH1+16* enhancer expression, we mutated the core nucleotides of the two SOXF motifs (see Materials and Methods for sequences) and tested the ability of the resultant *NOTCH1+16mutSOX-a/b* enhancer to drive reporter gene expression. Strikingly, mutation of the SOX motifs within the *NOTCH1+16* enhancer resulted in a dramatic reduction of reporter gene expression in endothelial cells in transgenic mice, although transgene expression was detected outside of the vascular system (Fig. 4A-C). This result was notably different from the *Dll4* enhancers, where mutations of SOXF motifs, or loss of SOXF factors, resulted in no detectable decrease on *Dll4* expression without the accompanying ablation of Notch signaling (Sacilotto et al., 2013).

Zebrafish *notch1b* is directly transcriptionally regulated by SoxF factors via an evolutionarily non-conserved enhancer

The SoxF-dependent *NOTCH1+16* enhancer robustly directed arterial-restricted expression in transgenic mouse models. However, this enhancer did not contain

sequence conservation beyond mammals (Fig. S1B), leaving it unclear how relevant these observations are to Notch signaling during arterio-venous specification in zebrafish, an extremely well-studied model system (Gore et al., 2012). We therefore investigated whether SoxF factors were able to transcriptionally regulate the zebrafish orthologue of NOTCH1, *notch1b*, by looking at the binding patterns of the zebrafish Sox18 transcription factor around the *notch1b* locus. Zebrafish Sox18, an orthologue of mammalian SOX18, is expressed in early endothelial cells and implicated in arterio-venous differentiation in conjunction with Sox7 (Cermenati et al., 2008; Hermkens et al., 2015; Herpers et al., 2008; Pendeville et al., 2008). Using 26-28 hours post fertilization (hpf) embryos from the *tg(fli1a:Gal4FF;10XUAS:Sox18mCherry)* zebrafish line, in which a tagged Sox18 was expressed specifically in endothelial cells (Fig. S3A), ChIP-seq analysis identified a Sox18 binding event 15-kb upstream of *notch1b* first exon (Fig. 5A). This binding peak was enriched in the enhancer-associated histone modifications H3K4me1 and H3K27Ac at 24hpf (Bogdanović et al., 2012; Kent et al., 2002) (Fig. 5A) suggesting that it may represent a novel enhancer of *notch1b*. A 1219-bp zebrafish DNA fragment corresponding to the Sox18-bound region, termed the *notch1b-15* enhancer, was cloned upstream of a silent *gata2a* promoter and GFP reporter gene within the Zebrafish Enhancer Detection (ZED) vector (Bessa et al., 2009) (Fig. 5B) and used to generate the stable *tg(notch1b-15:GFP)* fish line (Fig. 5C). The GFP transcript was detected in the vascular cord around the midline from 19hpf, and persisted in the vascular rod as it forms the dorsal aorta at 22hpf (Fig. 5C). GFP expression continued to be restricted to dorsal aorta and the segmental arteries in larvae from 24hpf until 48hpf. This arterial-restricted pattern of expression within the vasculature was similar to that of endogenous *notch1b* (Fig. S3B), indicating that the Sox18-bound *notch1b-15* element is a bona fide *notch1b* enhancer and suggesting that SoxF factors directly transactivate Notch receptor transcription in arterial endothelial cell in zebrafish.

ClustalW analysis comparing the orthologous enhancer sequences from fugu, stickleback and medaka revealed a remarkably similar pattern of conserved transcription factor motifs when compared to the *NOTCH1+16* enhancer (Fig. 6A), with multiple ETS and two SOX binding motifs, termed zfSOX-a and zfSOX-b, confirmed by EMSA analysis (Fig. 6B-C). To establish whether the zfSOX-a and zfSOX-b binding motifs were required for *notch1b-15* arterial enhancer function, we generated transient transgenic fish lines harboring mutated SOX binding motifs (*notch1b-15mutSOX-a/b:GFP*) and compared the activity of the transgene to WT

notch1b-15:GFP control transient transgenic animals (Fig. S4A-B). Simultaneous disruption of both zfSOX-a and zfSOX-b sites led to a reduction of arterial specific GFP expression in endothelial cells. While a minority of mutant fish still expressed GFP after SoxF binding sites mutation, the loss of expression was much greater than that seen after SoxF motif mutation in a previously published *Dll4* enhancer in transgenic zebrafish where vascular expression rates were unaffected by SOXFa/b mutation (Saciolto et al., 2013), supporting a key role for SOXF in *notch1b* activation. To further confirm our observation, we established stable transgenic *notch1b-15mutSOX-a/b:GFP* fish lines and compared them to the established WT *notch1b-15:GFP* lines. Analysis of the stable transgenic embryos (Fig. 7A) confirmed a GFP expression pattern in the dorsal aorta and segmental arteries for the WT transgene. By contrast, the *tg(notch1b-15mutSOX-a/b:GFP)* lines showed ectopic GFP expression in neurons and a significant decrease of GFP expression in the arterial endothelium (Fig.7A). Quantitative analysis of GFP intensity in both dorsal aorta and segmental arteries showed lower expression in most *tg(notch1b-15mutSOX-a/b:GFP)* embryos compared to WT *tg(notch1b-15:GFP)* larvae (Fig. 7B-C, Fig. S4C). Taken together these data clearly demonstrate that SOX-a/b binding sites are required to guide *notch1b-15* specific enhancer activity *in vivo* during arterial development.

We next investigated the consequences of morpholino-based (MO) knock-down of SoxF on *tg(notch1b-15:GFP)* fish. *sox7/sox18* double morphants exhibit a severe vascular phenotype (Fig. S5), including fusions and shunts between the dorsal aorta and cardinal vein (Cermenati et al., 2008; Herpers et al., 2008; Pendeville et al., 2008), phenotypes shared with *sox7;sox18* double mutant fish (Hermkens et al., 2015). MO-induced transcript depletion of *sox7* or *sox18* resulted in a down-regulation of *notch1b-15:GFP* expression, while double *sox7;sox18* morphant *tg(notch1b-15:GFP)* fish demonstrated a near-complete loss of reporter gene expression (Fig. 7D-E). Taken together, these results indicate that SoxF proteins directly modulate the activity of arterial-specific enhancers for both the mammalian NOTCH1 and zebrafish Notch1b receptors, positioning SoxF transcription factors directly upstream of Notch signaling during early arterial differentiation in both mammals and zebrafish.

Loss of endogenous *notch1b-15* enhancer activity perturbs Notch1b transcription and causes arterio-venous defects

To assess whether the endogenous *notch1b-15* regulatory element is functionally relevant during arterio-venous differentiation *in vivo*, we deleted the endogenous *notch1b-15* enhancer in zebrafish. The resultant *notch1b-15^{uq1mf}* allele was generated using two guide RNAs to drive rapid genome editing using the CRISPR/Cas9 system (Fig 8A), resulting in excision of a 203bp fragment overlapping the *notch1b-15* enhancer. Analysis of endogenous *notch1b* expression in the F₂ generation of the *notch1b-15^{uq1mf/uq1mf}* mutant line demonstrated lower *notch1b* expression levels in both DA and aISV of the *notch1b-15^{uq1mf/uq1mf}* homozygous embryos compared to their sibling controls, while no change was observed in the neural tube (Fig. 8B). Next, we assessed the *notch1b* transcript levels in purified *flt1*-positive arterial endothelial cell populations from F₃ generation of homozygous fish (F₂ *notch1b-15^{uq1mf}* homozygous in-cross) compared to wild type control larvae (F₂ *notch1b-15^{+/+}* x *flt1:YFP;lyve1:dsRed*) at 24-28hpf (Fig. 8C). As expected, *notch1b* transcripts were significantly down-regulated in the homozygous animals strongly supporting a role for the *notch1b-15* enhancer in the transcription of endogenous *notch1b* in arterial endothelial cells.

To assess the phenotypic outcome of *notch1b-15* loss of function, we took advantage of the *tg(notch1b-15^{uq1mf/+};flt1:YFP;lyve1:dsRed)* line to analyze the developing vasculature after *notch1b-15* enhancer deletion in both the F₂ and F₃ generations. Surprisingly given the reduced levels of *notch1b* (Fig. 8B), no overt vascular phenotype was detected in F₂ homozygous zebrafish *notch1b-15^{uq1mf/uq1mf}* (F₁ homozygous *notch1b-15^{uq1mf/uq1mf}* in-cross) (Fig. S6A), suggesting partial enhancer redundancy. Such redundancy, potentially explained by the pervasiveness of redundant, or “shadow”, enhancers around developmental genes (Cannavò et al., 2016), has been previously well documented in key endothelial genes, with examples including the *Dll4*, *Flk1* and *Tal1* loci (Cannavò et al., 2016). By contrast we detected a subpopulation (20-30%) of larvae from the F₃ generation (F₂ homozygous *notch1b-15^{uq1mf/uq1mf}* in-cross) that displayed a phenocopy of the *notch1b* loss of function phenotype as described in Fig. S6B. This increase in the phenotypic outcome in the F₃ generation is suggestive that in the context of the *notch1b-15^{uq1mf/+}* cross, mRNA maternal deposition likely contributes to compensate the disruption of *notch1b* transcription caused by the deletion of the *notch1b* enhancer, a compensation that is reduced in a purer *notch1b-15^{uq1mf/mf}* genetic background (Harvey et al., 2013).

To bypass potential rescuing effects of shadow enhancers or maternally deposited transcripts, we also investigated arterio-venous differentiation and sprouting

angiogenesis in zebrafish F₂ *notch1b-15^{uq1mfl/uq1mf}* after low-level depletion of *notch1b* mRNA levels. A splice *notch1b* morpholino (MO) was injected into eggs from a *notch1b-15^{uq1mfl/+}* in-crosses in the *tg(fltYFP;lyve1Dsred)* background. The *notch1b* MO was used at sub-optimal concentration (5ng/embryo) known to result in minimal phenotypes alone (Sacilotto et al., 2013). The developing vasculature of each resulting embryo was subsequently analyzed blindly at 3dpf, and genotypes were assigned to embryos after image acquisition (Fig. 8D, red). While most wild-type siblings had normal ISV development, we observed an increase number of hypersprouting ISVs across the *notch1b-15^{uq1mfl/+}* and *notch1b-15^{uq1mfl/uq1mf}* population (Fig. 8E, asterisks; 8F (top)) in a gene dosage dependent manner, similar to the phenotype described previously in high concentration *notch1b* morphant and Notch signaling deficient embryos (Geudens et al., 2010; Siekmann and Lawson, 2007). Further, mutant embryos also demonstrated loss of arterial connections between the dorsal aorta and dorsal longitudinal anastomotic vessel, similar to those described in Notch signaling deficient zebrafish (Geudens et al., 2010; Quillien et al., 2014); while the *notch1b* depleted *notch1b-15^{+/+}* and *notch1b-15^{uq1mfl/+}* embryos demonstrated an equal proportion of arterial and venous ISVs as previously reported (Bussmann et al., 2010) (Fig. 8E; 8F (bottom panel)).

To further confirm that interfering with *notch1b-15* enhancer activity is additive to *notch1b* transcripts depletion, we also chemically treated the *notch1b-15^{uq1mfl/+}* cross with *N*[(3,5-Difluorophenyl)acetyl]-L-alanyl-2-phenylglycine-1,1-dimethylethyl ester (DAPT), a well characterized Notch signaling inhibitor, over the course of endothelial differentiation (15-16ss through to 3dpf) (Fig. 8D, blue). All embryos treated with sub-optimal concentration of DAPT (5μM) showed a straight body axis, indicating that somitogenesis (and therefore Notch activity) was not significantly compromised (Fig. S7A). Interestingly, despite this lack of morphological defects, F₂ fish homozygous for *notch1b-15^{uq1mf}* allele displayed a lower arterial to total ISV ratio (Fig. 8G; S7A). On the other hand, fish homozygous for *notch1b-15^{uq1mf}* allele treated with DMSO vehicle alone had comparable aISV proportion to both sibling *notch1b-15^{+/+}* and *notch1b-15^{uq1mfl/+}* (Fig. S7B) similar to untreated control; suggesting that the observed loss of aISV is specific to an additive effect of DAPT treatment and *notch1b-15* enhancer activity disruption. Overall these data suggest a functional role of *notch1-15* enhancer in the endothelial-specific initiation of *notch1b* transcription to promote the acquisition of arterial cell identity.

SoxF factors are required for endogenous *Notch1* expression

Our results have clearly implicated SoxF factors as direct upstream regulators of arterial Notch enhancers, and therefore suggest a considerably greater role for SoxF alone in the regulation of the Notch receptors than in the Notch ligands. However, since the *notch1b-15* enhancer is partially redundant, we wanted to establish whether SOXF regulation was required for endogenous *Notch1/notch1b* expression itself, not just enhancer activity. Further, our results so far do not entirely rule out the possibility of SoxF/RBPJ combinatorial regulation of *Notch1/notch1b* as it was previously shown for *Dll4* enhancers (Saciotto et al., 2013). While neither human *NOTCH1+16* nor zebrafish *notch1b-15* enhancers contain conserved consensus RBPJ/Notch binding motifs, transcription factors can bind non-consensus motifs, and not all transcription factors necessarily bind conserved motifs (Wong et al., 2015). Lastly, the nature of the SoxF/Notch combinatorial regulation of *Dll4*, where the SOXF or RBPJ binding motifs played functionally interchangeable roles, this indicates potential direct interactions between these two proteins, meaning only a single SOXF binding motif may be necessary for SoxF/Rbpj synergy. Consequently, we investigated the consequences of SOXF depletion on endogenous *Notch1/notch1b* expression *in vivo*.

Although *Sox17* is robustly expressed in arterial endothelial cells (Corada et al., 2013; Hosking et al., 2009), compound *Sox7;Sox18* deletion in mice resulted in reduction of *Notch1* mRNA expression levels in the trunk dorsal aorta and primitive heart cavities of E8.5 embryos (Fig.9A, S8A). These results concur with observations in the mouse retina, where the vascular phenotype after triple *Sox7;Sox17;Sox18* endothelial-specific deletion closely resembled defects caused by loss of Notch signaling (Zhou et al., 2015). Strikingly, both MO-induced gene knock-down and compound mutation of *sox7* and *sox18* in zebrafish embryos also led to a near-complete loss of *notch1b* transcript expression specifically in endothelial cells as shown by *in situ* hybridization analysis (Fig. 9B-C, S8B). These results further establish an essential role for SoxF transcription factors in the induction of *Notch1* gene expression, and position SoxF proteins at the top of the transcriptional hierarchy regulating arterial specification.

Discussion

Recent work has implicated SOXF, ETS and RBPJ, the Notch transcriptional effector, in the regulation of the Notch ligand *Dll4* and many other key arterial genes (Corada et al., 2013; Lizama et al., 2015; Sacilotto et al., 2013; Wythe et al., 2013), but has not established the hierarchical arrangement of these diverse factors in arterial specification and differentiation. In this study, we demonstrate that arterial expression of the Notch receptor *Notch1/notch1b*, a key player in arterial specification, is directly downstream of SoxF regulation in both fish and mouse. Unlike with other key arterial specification markers, including *Dll4*, *Efnb2a* and *Dlc* (Sacilotto et al., 2013), ablation of *Notch1/notch1b* expression after depletion of SoxF factors occurred without concurrent inhibition of Notch signaling. Therefore, this work positions SoxF factors directly above Notch signaling in the transcriptional hierarchy initiating arterial development, and suggests that SoxF factors may initiate a feed-forward loop directing arterial identity. In this model, SoxF factors would first activate Notch signaling via the transcriptional activation of Notch receptors in combination with weak activation of Notch ligands (Sacilotto et al., 2013). This early SoxF-mediated activity would then be boosted by the initiation of Notch signaling, resulting in the sustained activation of other downstream genes, eventually activating the full cohort of genes necessary to acquire and maintain arterial endothelial cell identity.

Understanding the function of SoxF factors through mutational analysis has presented significant challenges. Strain-specific variations in mice after depletion of separate SoxF genes, and varying levels of compensation from other SoxF factors have resulted in some contradictory reports (Corada et al., 2013; Lee et al., 2014), as is also the case for MO-based zebrafish analysis (Cermenati et al., 2008; Herpers et al., 2008; Pendeville et al., 2008). Nonetheless, the results described here agree with an increasingly convincing body of work suggesting that SoxF factors influence Notch signaling yet are unaffected by Notch ablation. For example, over-expression of Sox17 up-regulates components of the Notch pathway (Corada et al., 2013; Lizama et al., 2015), loss of functional SoxF factors results in defects similar to those observed after Notch inhibition in mice and fish (Corada et al., 2013; Sakamoto et al., 2007; Zhou et al., 2015), while Notch ablation results in little alteration to the endothelial expression of SoxF factors in both mice and fish (Abdelilah et al., 1996; Corada et al., 2013). Data reported here combines with these reports to strongly support the role for SoxF factors as part of the initial transcriptional machinery that instructs arterial specification events. However some questions remain. In particular,

it is notable that double *sox7;sox18* mutant fish, although exhibiting severe arterio-venous defects very similar to those seen in Notch-deficient fish (Lawson et al., 2001), do not fully recapitulate the effects of *Vegfa* depletion on arterial specification (Lawson et al., 2002). While part of this difference may be attributed to a weak expression of zebrafish *sox17*, which is expressed in some arterial endothelial cells (Hermkens et al., 2015), it is also expected that the *Vegfa* pathway has a wider effect on arterial endothelial cells more generally, away from SoxF-mediated activation of Notch signaling. SoxF factors are also influenced by signaling pathways beyond *Vegfa*. The diverse nature of roles for *Vegfa* in the vasculature, including the regulation of both sprouting angiogenesis and arteriogenesis, processes that inevitably involve different cohorts of downstream targets, make it necessary that multiple regulatory pathways interact with *Vegfa* during vascular development. While recent work has shown that *Vegfa* signalling increases the nuclear translocation of SoxF (Duong et al., 2014), and inhibition of *Vegfa* results in the loss of vascular *sox7* in fish, *sox18* is still expressed in the absence of intact *Vegfa* signaling (Pendeville et al., 2008). Additionally, the loss of the *Vegf* co-receptor *Nrp1* has little effect on SoxF expression in mouse retinal vasculature (Zhou et al., 2015), pointing to other upstream influences on SoxF function in endothelial cells. Other upstream effectors of SoxF function are likely to include canonical Wnt signaling and *Vegf-d*, both of which have been shown to influence SoxF factors (Corada et al., 2013; Duong et al., 2014; Zhou et al., 2015).

Recent evidence has also implicated a role for a coordinated *Vegf*-Mapk-Ets pathway in the induction of Notch signaling components and early arterial differentiation (Wythe et al., 2013). Notably, in addition to SoxF motifs, both the *Notch1* and *Dll4* enhancers share a number of highly conserved consensus motifs for the Ets family of transcription factors. While ETS motifs are common to all vascular enhancer elements, including many that are not preferentially expressed in the arterial vasculature (De Val and Black, 2009), the Ets factor *Erg* has been specifically implicated in arterial-specific regulation of *Dll4* (Wythe et al., 2013). It is therefore likely that *Vegfa*-mediated activation of Ets factors may contribute to the transcriptional activity of Notch downstream effectors, and thus influence arterial establishment independent of SoxF factors. However, it is notable that *Vegfa*-mediated Ets transcription does not appear to be sufficient for arterial gene expression. *Dll4* enhancers lacking SOXF and RBPJ motifs but retaining all ETS motifs were unable to drive any transgene expression (Sacilotto et al., 2013), nor were Notch enhancers lacking SOXF motifs (Fig 2-4 of this study). Similar results

were found in other delineated arterial enhancers, including the Ece1 upstream enhancer (Robinson et al., 2014), and the Flk1 intron10 enhancer, where loss of Rbpj-mediated repression resulted in expansion of enhancer activity into venous cells without alterations to ETS motif binding (Becker et al., 2016). Combined with recent observations demonstrating that Erg also plays a crucial role in venous specification through activation of *Ap1nr* (Lathen et al., 2014), it is therefore likely that the role of Erg, and other Ets factors, downstream of Vegfa on arterial-restricted gene expression occurs in co-operation with other essential, arterial-specifying transcription factors. The data presented in this work, combined with the analysis of other arterial enhancers including Dll4 and Ece1 (Robinson et al., 2014; Sacilotto et al., 2013; Wythe et al., 2013), increasingly suggest that SoxF may fulfill this role.

Materials and Methods

Cloning

The 10XUAS:Sox18mCherry plasmid DNA was generated using the full-length mouse *Sox18* cDNA sequence, tagged with 10xUAS and mcherry and cloned into pDestTol2CG2. The *notch1b-15:GFP* WT were generated by PCR from zebrafish genomic DNA and cloned into the Zebrafish Enhancer Detector (ZED) vector (Bessa et al., 2009). *notch1b-15mutSOX-a/b* was generated by site-directed PCR mutagenesis of the WT construct. The *NOTCH1-68*, *NOTCH1+3/5*, and *NOTCH1+33* enhancers were generated by PCR from mouse genomic DNA, *NOTCH1+16* WT and *NOTCH1+16mutSOX-a/b* enhancers were generated as custom-made, double-stranded linear DNA fragments (GeneArt® Strings™, Life Technologies). All mammalian fragments were subsequently cloned into the hsp68-LacZ-Gateway vector (provided by N. Ahituv) (De Val et al., 2004).

Transgenic animals and genome editing

Where appropriate, animal procedures were approved by local ethical review and licensed by the UK Home Office, or conformed to institutional guidelines (University of Queensland Animal Ethics Committee). Transgenic mice were generated by oocyte microinjection and analysed as described previously (De Val et al., 2004). Compound *Sox7^{-/-};Sox18^{-/-}* (C57BL/6) mouse embryos were generated on the C57BL/6 background through crossing heterozygous *Sox7:tm1* to *Sox18:tm1* generating *Sox7^{+/-};Sox18^{+/-}* mice which were subsequently in-crossed (Pennisi et al., 2000a). Transgenic zebrafish embryos were generated using the Tol2 system in conjunction with the ZED vector (Bessa et al., 2009). The *sox7^{hu5626}; sox18^{hu10320}* double homozygous mutant zebrafish are as previously described (Hermkens et al., 2015). The *tg(fli1a:Gal4FF,10XUAS:Sox18mCherry)* zebrafish line was generated by crossing *10XUAS:Sox18mCherry* with *fli1a:Gal4FF, 4XUAS Utrophin GFP*. Morpholino-mediated knockdown was as previously described (Herpers et al., 2008). CRISPR genome editing for *notch1b-15* was performed as described in Gagnon et al., 2014 to generate *notch1b-15^{uq1mf}* (203-base-pair (bp) deletion) allele. The *F₂ notch1b-15^{uq1mf/1mf}* was generated by in-crossing *notch1b-15^{uq1mf/+}* while the *F₃ notch1b-15^{uq1mf/1mf}* was generated from the *F₂ notch1b-15^{uq1mf/1mf}* in-crossed, both of the *tg(fltYFP;lyve1Dsred)* background.

Chromatin immunoprecipitation

Positive *tg(fli1a:Gal4FF;10XUAS:Sox18mCherry)* fish larvae were collected at 26-28hpf and treated as previously described (Mohammed et al., 2013). DNA amplification was performed using TruSeq ChIPseq kit (Illumina, IP-202-1012) following IP. The library was quantified using the KAPA library quantification kit for Illumina sequencing platforms (KAPA Biosystems, KK4824) and 50bp single end reads were sequenced on a HiSeq2500 following the manufacturer's protocol. Illumina fastq files were mapped to GRCz10/danRer10 genome assembly using bowtie, and peaks were called using MACS version 2.1.0. using input as a reference. To avoid false positive peaks calling due to the mCherry epitope, ChIP-seq with the mCherry epitope only were performed in parallel to Sox18-mCherry ChIP-seq and peaks called in these experimental conditions were subtracted to the peaks called in the Sox18-mCherry conditions.

Motif identification and EMSA

The sequences were analyzed for consensus sequence motifs by eye, and using the TRANSFAC® program (www.biobase-international.com/transcription-factor-binding-sites) from BIOBASE Corporation (Matys et al., 2006). Electrophoretic mobility shift assay (EMSAs) were performed as described previously (De Val et al., 2004).

Morpholinos and drug treatment

Morpholino-mediated knockdown was as previously described. ATG Morpholinos against *sox7* and *sox18* were injected into 1-2 cell *tg(notch1b-15:GFP)* stable line at a concentration of 5ng/embryo (Herpers et al., 2008). To assess the effect of *sox7/18* knockdown on endogenous *notch1b* transcripts, *sox7* and *sox18* MO were injected into wild-type zebrafish larvae at 1ng/embryo in parallel with a standard control MO (std-MO) injected at 2ng/embryo (Cermenati et al., 2008). To characterize *notch1b-15^{uq1mf}*, MO-*notch1b* were injected into the *uq^{1mf/+}* cross at 5ng/embryo.

For *N*-[(3,5-Difluorophenyl)acetyl]-L-alanyl-2-phenylglycine-1,1-dimethylethyl ester (DAPT) treatment, we used 5μM dissolved in 1% DMSO. Fish were treated from 15-16 somite stage to 3dpf and media + DAPT was refreshed daily during experiment.

In situ hybridization and immunofluorescence staining.

Wholemout *in situ* hybridization in zebrafish larvae was performed as described (Coxam et al., 2015; Duong et al., 2014). Section and wholemount mouse *in situ* was performed as described (Metzis et al., 2013; Fowles et al., 2003). The *Notch1* probe was generated by PCR from mouse embryo cDNA pool at 14.5dpc, and reverse

transcribed with T7 polymerase. Wholemount immunohistochemistry for anti-GFP was performed as described (Koltowska et al., 2015).

Quantification and data analysis

To characterize the vasculature in Fig. 8E-G, Fig. S6-S7, intersomitic vessels (20-22 ISVs) expressing *tg(flt1YFP)* were analyzed across 10-11 somites through a Z-stack using ImageJ (NIH Image, USA) after image acquisition by confocal microscopy. Intersomitic vessels connecting the DLAV to the dorsal aorta expressing YFP were assigned as arterial ISVs. Vessels were also scored for ectopic sprouting. The proportion of aISVs or hypersprouts to total number of ISVs was subsequently analyzed by two-tailed Mann-Whitney U test. To quantify the GFP intensity of *tg(notch1b-15:GFP)* and *tg(notch1b-15mutSox-a/b:GFP)* (Fig7A), 2-3 ISVs across 5-6 somites in the trunk region were analyzed using Image J. A region of interest (ROI) covering a single ISV was selected and mean pixel intensity for each ISV was quantified from each individual stack across 3 z-sections. This value was further corrected by subtracting the background value. Average ISV GFP intensity (quantified from 2-3 ISVs) for each fish larva was subsequently corrected for its genomic GFP copy number. Similar method was used to quantify GFP intensity in the endothelial lining along the dorsal aorta.

Fluorescent Activated Cell sorting (FACS) and expression analysis

FltYFP positive endothelial cells were isolated from WT and F₃ *notch1b-15^{uq1mf/1mf}* at 24-28hpf. RNA was extracted, amplified and cDNA was synthesized as previously described (Coxam et al., 2014; Picelli et al., 2014). Primer sequences and detailed analysis for qPCR are in the Supplemental Material.

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Competing interests

No competing interests declared

Author contributions

Conceptualization, I.C., M.Frit., S.D.V. and M. Fran.; Methodology, I.C., M.Frit., S.D.V. and M. Fran.; Formal analysis, K.H. and J.C.; Investigation, I.C., M.Frit., C.P-T., A.N. K.H., A.L., J.O., D.D., A.O., D.H., K.L. I.R., M.C., B.H.; Resources, A.L., G.B.-G., J.C., S.S-M.,M.B.; Data Curation K.H. and J.C.; Writing-original draft, I.C., S.D.V, M. Fran.; Writing-review and editing, I.C., B.H., M.B., S.D.V, M. Fran.; Visualization, I.C., S.D.V and M.Fran.; Supervision, G.B.-G., J.C., E.D., B.H., M.B., S.D.V., M.Fran.; Project Administration, S.D.V. and M.Fran.; Funding Acquisition, S.D.V., M.B. and M.Fran.

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Figure Legends

Figure 1. The human *NOTCH1* locus contains multiple putative endothelial enhancers.

(A) Schematic representation of the human *NOTCH1* locus from UCSC ENCODE Browser (<http://genome.ucsc.edu>). HUVEC specific H3Me1 and H3K27Ac (enhancer associated) and H3K4Me3 (promoter associated) peaks are denoted in light blue (both in the separate HUVEC and combined tracks (denoted as 'all')), other colors indicate H3K4Me1, H3K4Me3 and H3K4Ac peaks specific to non-endothelial cell lines (GM12878 cells (red), H1-hESC cells (yellow), HSMM cells (green), K562 cells (purple) NHEK cells (lilac) and NHLF cells (pink). Two other genes are denoted within this browser image, INPP5E and SEC16A, in blue text, *NOTCH1* gene denoted in black text. DNase I digital hypersensitive hotspots are indicated by black vertical lines on grey (HUVEC, HMVEC-dBI-Ad, HMVEC-dBI-Neo and HMVEC-LBI, all different endothelial cell types). The four *NOTCH1* putative enhancer regions were identified by high levels of HUVEC-specific H3K4Me1 and H3K27Ac associated with endothelial cell-specific DNase I hypersensitivity hotspots. These putative enhancer regions are indicated by thick black horizontal bars, putative promoters identified by H3K4Me3 are indicated by red triangles and red P.

(B) Zoomed representation of the human *NOTCH1* gene (upper, in 5' to 3' orientation with putative enhancers indicated) and *NOTCH1+16hsp* transgene (lower).

(C) Summary of the reporter gene expression patterns detected in E12 mice transgenic for each putative *NOTCH1* enhancer region.

Figure 2. The *NOTCH1+16* transgene directs arterial endothelial cell-restricted expression in transgenic mice.

(A-N) Representative transgenic whole-mount embryos (A-H) and transverse sections (I-N) show *LacZ* reporter gene expression (gene product β -galactosidase detected by blue X-gal staining) in arterial endothelial cells throughout embryonic development.

(O-P) E12 transverse paraffin section shows that expression of the venous marker Endomucin (Emcn) did not overlap with the *LacZ* reporter gene on the same section.

Figure 3. The *NOTCH1+16* enhancer contains SOXF and ETS binding motifs.

(A) Multispecies alignment of the orthologous region of the *NOTCH1+16* enhancer from human, mouse and opossum (oposs) using ClustalW. Colored sequences are

confirmed by EMSA; gray sequences are motifs identified *in silico* that did not bind in EMSA.

(B) Radiolabeled oligonucleotide probes encompassing NOTCH1+16 SOX-a (lanes 1-8) and SOX-b (lanes 9-16) were bound to recombinant SOX7 (lanes 2-4 and 10-12) and SOX18 (lanes 6-8 and 14-16). Both proteins efficiently bound labeled probes (lanes 2, 6, 10 and 14), were competed by excess unlabeled self-probe (WT, lanes 3, 7, 11 and 15) but not by mutant self-probe (MT, lanes 4, 8, 12 and 16).

(C) Radiolabeled oligonucleotide probes encompassing NOTCH1+16 ETS-a (lanes 17-20), ETS-b (lanes 21-24) and ETS-c (lanes 25-28) were bound to recombinant ETV2 proteins. All proteins efficiently bound to labeled probes (lanes 18, 22 and 26), were competed by excess unlabeled self-probe (WT, lanes 19, 23 and 27) but not by mutant self-probe (MT, lanes 20, 24 and 28).

Figure 4. SOXF factors are required for *NOTCH1+16* expression.

(A) Summary of reporter gene expression detected in E12 transient transgenic mice. * indicates faint vascular expression detected during in section analysis as indicated but not visible in whole-mount analysis.

(B) Four independent whole-mount representative E12 X-gal stained embryos transgenic for the *NOTCH1+16mutSOX-a/b* construct. Numbers on the bottom left indicate the unique embryo identifier.

(C) Transverse sections taken from two *NOTCH1+16mutSOX-a/b* embryos demonstrate the very limited endothelial expression was detected in these embryos (asterisks).

Figure 5. A Sox18-bound region within the *notch1b* locus represents a bona-fide arterial specific enhancer.

(A) Schematic representation of part of the zebrafish *notch1b* locus from UCSC ENCODE Browser (<http://genome.ucsc.edu>). *notch1b* gene is in 3' to 5' orientation, H3K27Ac peaks at 24hpf are in purple, H3K4me1 at 24hpf peaks are blue, SOX18 ChIP-seq peaks are red, region encompassing the *notch1b-15* enhancer is purple horizontal bar.

(B) Schematic representation of the ZED *notch1b-15:GFP* transgene. Ins indicates insulator sequences, GATA2 prom indicates the silent GATA2 promoter, ZED control:RFP indicates the active cardiac actin enhancer/promoter construct fused to the RFP gene used as a positive control in the ZED vector.

(C) The *notch1b-15:GFP* transgene directs arterial endothelial cell-specific expression in transgenic zebrafish line *tg(notch1b-15:GFP)*. Representative

transgenic whole-mount embryos and transverse sections show reporter gene expression, as detected by *in situ* hybridization (top row, blue) or GFP fluorescence (bottom two rows, green) in arterial endothelial cells throughout embryonic development. nt = neural tube, nc = notochord, DA = dorsal aorta, PCV = posterior cardinal vein, aISV (white arrows) = arterial intersomitic vessel, DLAV = dorsal longitudinal anastomotic vessel.

Figure 6. The *notch1b-15* enhancer contains essential SOXF binding motifs.

(A) Multispecies alignment of the orthologous regions of the *notch1b-15* enhancer from zebrafish (zfish), fugu, stickleback (stickle) and medaka using ClustalW. Colored sequences are confirmed by EMSA; gray sequences are motifs identified *in silico* that did not bind robustly in EMSA.

(B) Radiolabeled oligonucleotide probes encompassing *notch1b-15* zfSOX-a (lanes 1-8) and zfSOX-b (lanes 9-16) were bound to recombinant SOX7 (lanes 2-4 and 10-12) and SOX18 (lanes 6-8 and 14-16). Both proteins efficiently bound labeled probes (lanes 2, 6, 10 and 14), were competed by excess unlabeled self-probe (WT, lanes 3, 7, 11 and 15) but not by mutant self-probe (MT, lanes 4, 8, 12 and 16).

(C) Radiolabeled oligonucleotide probes encompassing *notch1b-15* zfETS-a (lanes 17-20) and zfETS-b (lanes 21-24) were bound to recombinant ETV2 proteins. All proteins efficiently bound to labeled probes (lanes 18 and 22), were competed by excess unlabeled self-probe (WT, lanes 19 and 23) but not by mutant self-probe (MT, lanes 20 and 24).

Figure 7. SOXF factors are required for *notch1b-15* expression.

(A) Confocal projection of stable transgenic wildtype *tg(notch1b-15:GFP)* (left) and *tg(notch1b-15mutSOX-a/b:GFP)* (right), where both zfSOX-a and zfSOX-b sites have been simultaneously mutated. Representative larvae from 4 independent founders were shown. Asterisks indicated the reduced GFP expression in the dorsal aorta. White arrowheads showed the ectopic neuronal expression observed in *tg(notch1b-15mutSOX-a/b:GFP)*.

(B-C) Graph indicating the intensity of GFP expression in the dorsal aorta and arterial intersomitic vessels of the stable transgenic *tg(notch1b-15:GFP)* and *tg(notch1b-15mutSOX-a/b:GFP)* at 2dpf. Expression is normalized to GFP genomic copy number. Fish were pooled from 3-4 separate founders. Mean \pm SEM; scored *tg(notch1b-15)*, n=41; *tg(notch1b-15mutSOX-a/b:GFP)*, n=29; Mann-Whitney. (****) $p < 0.0001$.

(D) Graph indicating levels of GFP expression in *tg(notch1b-15:GFP)* zebrafish embryos after morpholino injection. Number of fish for each condition was indicated.

(E) Representative examples of *tg(notch1b-15:GFP) sox7/18* double morphant zebrafish at 24hpf compared to un-injected controls. Double morphants demonstrated reduced EGFP expression in the dorsal aorta and intersomitic vessels.

Figure 8. Loss of endogenous *notch1b-15* compromised arterial formation and reduces endogenous *notch1b* transcript level.

(A) Schematic representation showing the deleted region of *notch1b-15* mutant allele *uq^{1mf}*, which includes both *zfsOX-a* and *zfsOX-b* sites (yellow).

(B) The F_2 *notch1b-15^{uq1mf/uq1mf}* has reduced *notch1b* expression in dorsal aorta and intersomitic vessels (white arrows) compared to the sibling wild-type and Hets (black arrows) at 26-28hpf.

(C) Quantitative PCR on FAC-sorted endothelial populations at 24-28hpf showed that F_3 *notch1b-15^{uq1mf/uq1mf}* fish has lower *notch1b* expression compared to wild-type fish. Expression is relative to *kdrl* and *flt1*. Mean \pm SEM; n=6 (*uq1mf/uq1mf*) and 8(WT) independent sorts, where each sorts was pooled from 60-100 larvae; t-test. (*) $p < 0.05$; (***) $p < 0.001$.

(D) Schematic showing the treatment conditions conducted to characterize the vascular phenotype of *uq^{1mf/+}* cross. In Treatment 1 (T1, red), *notch1b* morpholino was injected at 1-2 cell stage. The developing vasculature of each embryo was then analyzed blindly at 3dpf. After scoring genotypes were assigned to each larva. In Treatment 2 (T2, blue), larvae from the *uq^{1mf/+}* cross were treated with/without DAPT (5 μ M) from 15-16hpf until 3dpf. Vessels of these treated larvae were blindly scored prior to genotyping as reported for T1.

(E) At 3dpf, *notch1b-15^{uq1mf/uq1mf}* *notch1b* morphants frequently showed ectopic sprouting in between the intersomitic vessels (asterisks) compared to sibling wild-type *notch1b* morphants. Mutants also have loss of arterial connections (red arrowheads) between the dorsal longitudinal anastomotic vessel (DLAV) and dorsal aorta (DA), as indicated by the loss of YFP expression in *tg(fltYFP)* background.

(F) (Top panel) Quantification of hyper-sprouting ISV number in individual *notch1b* morphants labeled by *tg(fltYFP)* at 3dpf. Mean \pm SEM; scored sibling WT (+/+), n=20; Het (*uq^{1mf/+}*), n=32; Hom (*uq^{1mf/1mf}*), n=21; Mann-Whitney. (**) $p < 0.005$. (Bottom panel) Quantification of YFP positive intersomitic vessels which connect between DLAV and DA in individual *notch1b* morphants labeled by *tg(fltYFP)* at 3dpf. Mean \pm SEM; scored sibling WT (+/+), n=20; Het (*uq^{1mf/+}*), n=32; Hom (*uq^{1mf/1mf}*), n=21; Mann-Whitney. (**) $p < 0.005$.

(G) Quantification of YFP positive intersomitic vessels that connect between DLAV and DA in individual embryos treated with 5 μ M DAPT at 3dpf. Vessels are labeled by *tg(fltYFP)*. Mean \pm SEM; scored sibling WT (+/+), n=9; Het (*uq*^{1mf/+}), n=25; Hom (*uq*^{1mf/1mf}), n=14; Mann-Whitney. (*) p<0.05.

Figure 9. Mouse and zebrafish arterial *Notch1* expression is dependent on SOX7/18 activity.

(A) Cross-sections on wholemount *in situ* hybridization for *Notch1* transcript on E8.5 mouse embryos shows reduction of *Notch1* expression (asterisks) in the bulbus cordis region of the primitive heart and vitelline artery of *Sox7/Sox18* double knock-outs. bc = bulbus cordis, va = vitelline artery.

(B) At 24hpf *notch1b* expression is significantly down-regulated in the dorsal aorta (asterisks) of *sox7/sox18* double morphants, while its signal is unaffected in the neural tube. *flt1* expression is comparable between controls and *sox7/sox18* double morphants indicating that the dorsal aorta is correctly formed.

(C) *notch1b* was barely detected in the dorsal aorta and ISVs of *sox7/18* double knock-outs zebrafish (asterisks) compared to the wildtype or *sox7* or *sox18* hets (black arrows).

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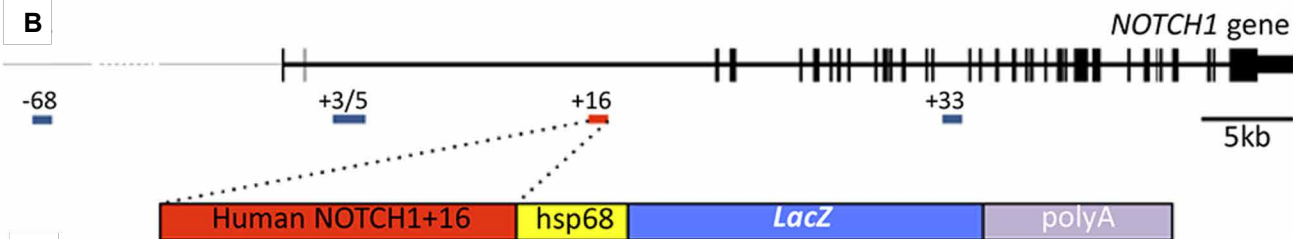
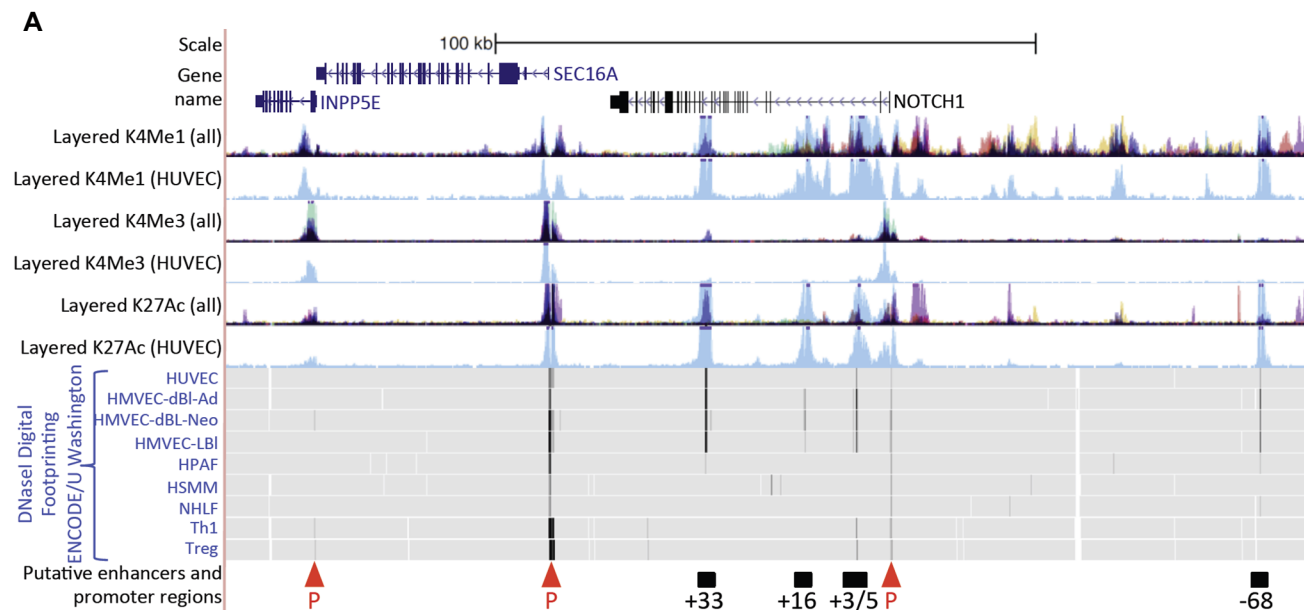
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C

Transgene	Number of tg mice	Tg mice with any detectable X-gal expression	Tg mice with X-gal expression in arterial endothelial cells	Tg mice with X-gal expression in venous endothelial cells
NOTCH1-68	5	3	0	0
NOTCH1+3/5	4	4	0	0
NOTCH1+16	10	9	6	0
NOTCH1+33	5	5	1	1

Figure 1

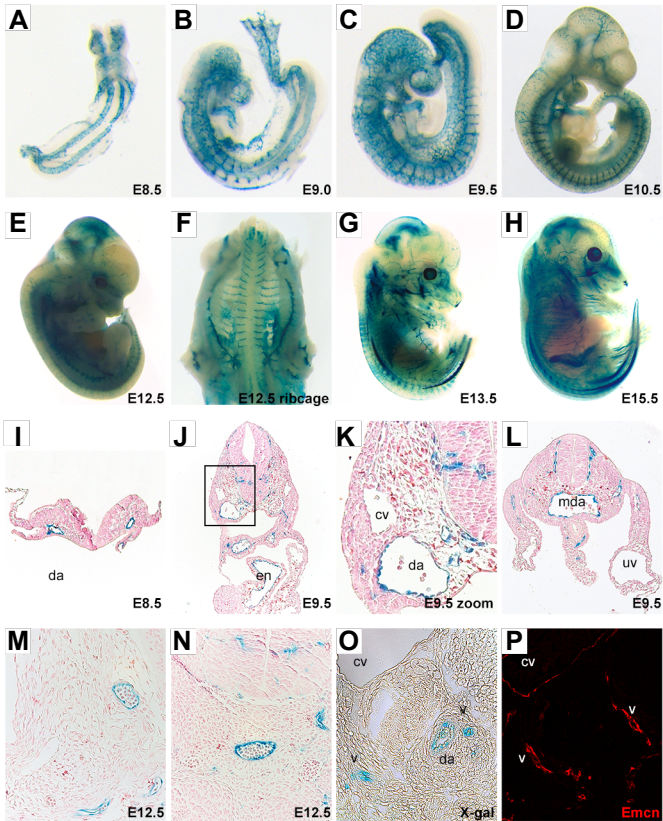


Figure 2

A

hmETS-a hmETS-nb hmETS-b hmETS-c

human CAAATCTTTCC CCC-AGTTGCATCCTCGGATGGTTTAAATGTGC-----GAAGGAGAA GTGCAAGAGGC--

mouse CAAATCT- TTCCCCACAGTTGCATCCTTGGGTGGTTTAAATACGCT-----GAAAAAGAA GCTCAAGAAAG--

oposs TAAGTGACATCCT--TAGAGGAGTTTAACTGTTGACCCTCCTCCACTGGGAAGGAGAAATGTAAGAAACAA

** * **** ** * * * ** * * * * * ** * **** * ****

hmETS-nb hmETS-nb hmSOX-a

human C--AGGTTTCTCTCTGGCTC-----CGGGAAACCTTTGTGTGG-GAG

mouse CGCAGGCTTCTTGGCTGGCTC-----AGGGAAGCCCTTTGTGTGG-GAG

oposs ACACACCTTCTCTCCAGCAAAATGTGGTGGCTTGCCAGCCTGAGAAAAGAGGAGAAATCCCTTTGTGCAGTGAG

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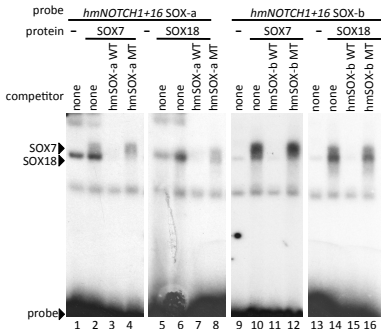
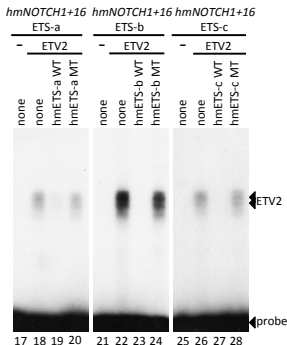
hmETS-nb hmETS-nb hmETS-nb hmSOX-b

human GCGGGATGGGGA-----CAGAACCTGTCTCCCGGGAAGCTACC-TTCCCGCAGCCGGGAAACAATGCCCTCA

mouse ACAGT-TGAAGT-----TGGAACCTGCCCTTAGGGGAAGCCACCTTTTCCCACTATAGGGAAACAATACTCCCC

oposs AATGGATGAGGAATGAGCTAGGAATGGTGATCTCTGGAAGCCTGT-TTCCCACTATCATGAAACAATGCCTTAA

** * * * * ** * * * * * * * * * * * *

B**C****Figure 3**

A

Transgene	Number of tg mice	Tg mice with any detectable X-gal expression	Tg mice with X-gal expression in arterial endothelial cells	Tg mice with X-gal expression in venous endothelial cells
<i>NOTCH1+16</i> WT	8	7	4	0
<i>NOTCH1+16 mutSOX-a/b</i>	9	6	0*	0*

B

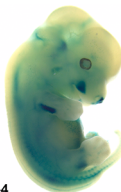
NOTCH1+16mutSOX-a/b E12 embryos



1-17



1-24



2-4



2-7

C

NOTCH1+16mutSOX-a/b embryo 1-17

NOTCH1+16mutSOX-a/b embryo 2-4

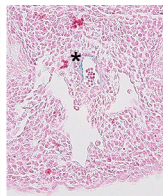
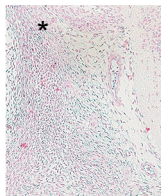
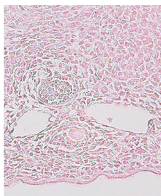
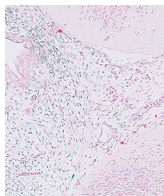


Figure 4

A

UCSC Genome Browser on Zebrafish Jul. 2010 (Zv9/danRer7) Assembly

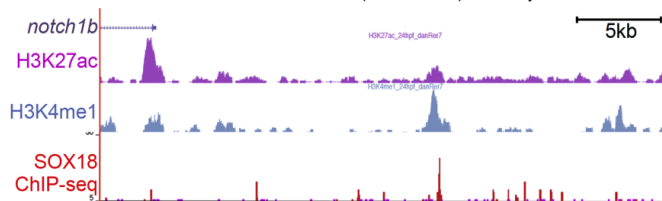
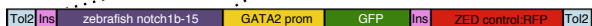
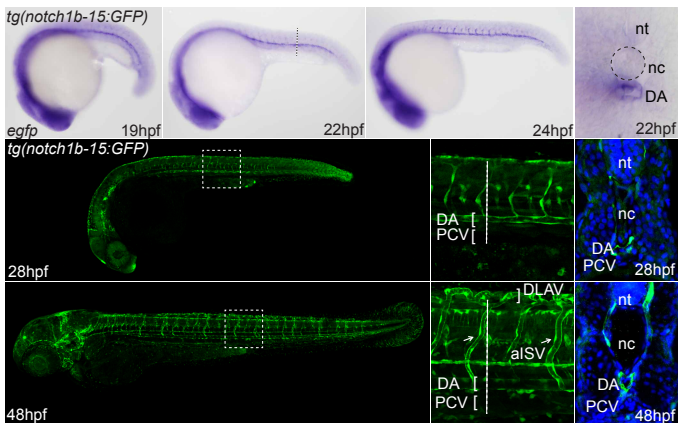
**B****C**

Figure 5

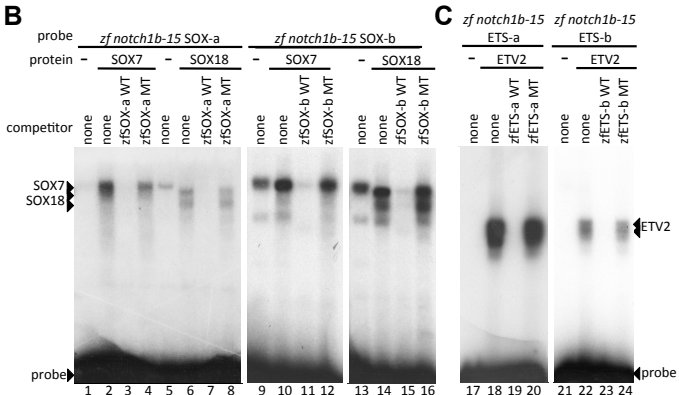
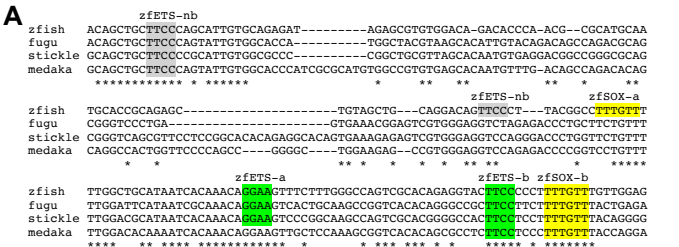


Figure 6

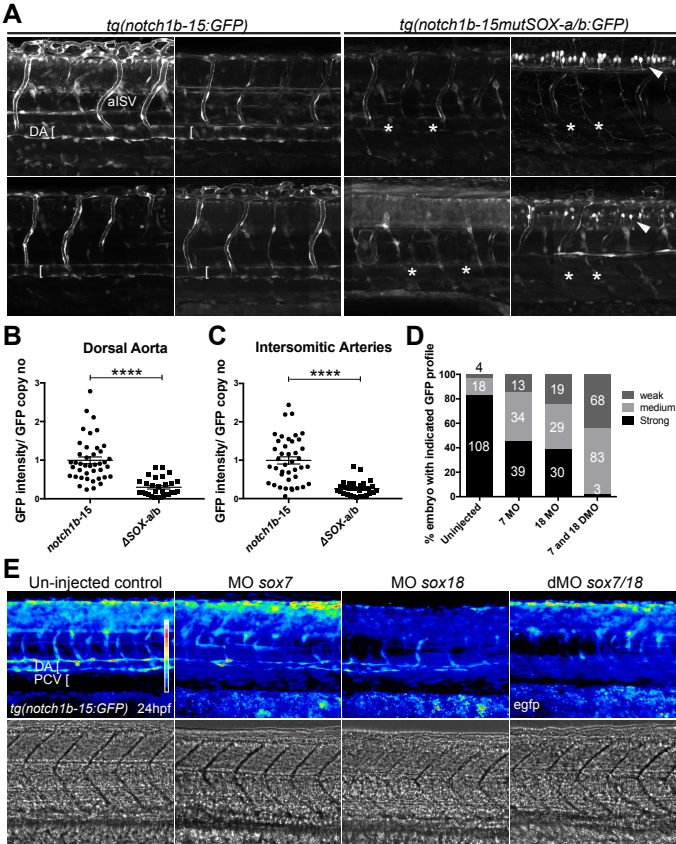


Figure 7

WT zfish
notch1b-15^{eq1af} CAGCTGCTCTCCAGCAGATGTGTCAGAGATAGAGCGCTGTGGACAGACACCCAAAGCGGCATGCAATGCACCCGACAGAGCTGTAG

WT zfish
notch1b-15^{eq1af} CTGCTGCTCTCCAGCAT zFETS-nb zFSOX-a zFETS-a

WT zfish
notch1b-15^{eq1af} CAGCAGGACAGTCTCCCTTTCGGCCCTTTGCTTTGCTGCATAATCAACAAGAGAGTTCCTTTGGGCGCAGTCGCGACAGA

WT zfish
notch1b-15^{eq1af} zFETS-b zFSOX-b

WT zfish
notch1b-15^{eq1af} GGTGATCTCCGCTTTTGTTGTTGGAGGTTGTGTGAAAGTTTGTGGTACAGACCCACAGTCGCGCCCAATTCTCTTCATTC

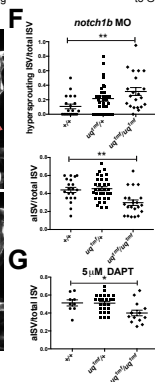
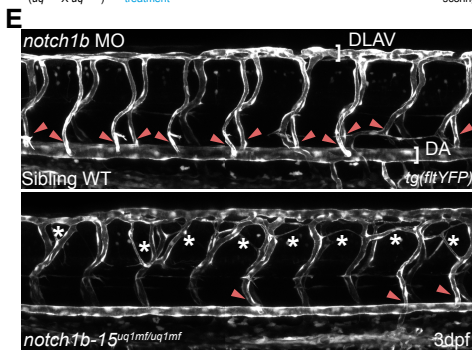
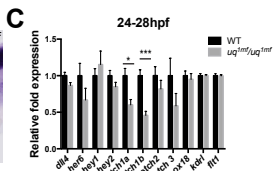
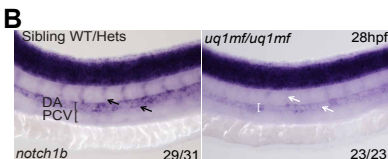


Figure 8

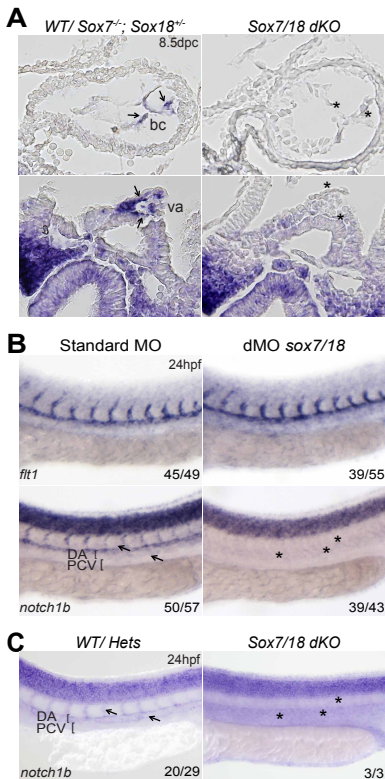


Figure 9